J. Indonesian Trop. Anim. Agric.
40(2):93-99, June 2015
pISSN 2087-8273 eISSN 2460-6278

NUCLEAR MATURATION RATE OF SHEEP OOCYTES *IN VITRO*: EFFECT OF STORAGE DURATION AND OVARY TEMPERATURE

A. Febretrisiana[1], M. A. Setiadi[2] and N. W. K. Karja[2]
[1]Research Institute for Goat Production, PO Box I Sei Putih, Galang 20585, North Sumatera - Indonesia
[2]Faculty of Veterinary Medicine, Bogor Agricultural University, Jln. Agatis, Darmaga Campus, Bogor 16680 - Indonesia
Corresponding E-mail: febre_arie@yahoo.com

Received November 06, 2014; Accepted March 19, 2015

ABSTRAK

Penelitian ini bertujuan untuk mengetahui pengaruh suhu dan waktu penyimpanan ovarium terhadap tingkat maturasi inti oosit secara *in vitro* pada domba. Ovarium diperoleh dari rumah potong hewan dan disimpan dalam medium NaCl fisiologi dengan lama waktu penyimpanan yang berbeda (2-4 jam, 5-7 jam dan 8-10 jam setelah pemotongan hewan) dan temperatur penyimpanan yang berbeda (27-28°C, 36-37°C dan 4°C). Oosit dengan sel-sel kumulus yang kompleks dikoleksi masing-masing dari ketiga kelompok suhu dan waktu penyimpanan dengan metode *slicing* dan kemudian dimaturasi selama 26 jam. Tidak ada perbedaan tingkat maturasi inti oosit yang mencapai tahap metafase II pada penyimpanan suhu 27-28°C maupun pada suhu 36-37°C dengan waktu penyimpanan selama 2-7 jam (P>0,05). Sedangkan tingkat maturasi yang lebih rendah ditunjukkan oleh oosit yang dikoleksi dari ovarium yang disimpan pada suhu 4°C (69,23%, 70,83% dan 45,65% berturut-turut untuk waktu penyimpanan 2-4 jam dan 59,61%, 64,58% dan 36,36% berturut-turut untuk waktu penyimpanan 5-7 jam. Tingkat maturasi dari kelompok penyimpanan dengan suhu 27-28°C dan 36-37°C mengalami penurunan dan sebaliknya penyimpanan pada suhu tidak menunjukkan penurunan tingkat maturasi oosit setelah penyimpanan 8-10 jam (24,37%, 7,84% dan 45,23%, berturut-turut) (P<0,05). Tingkat maturasi oosit pada suhu 4°C justru lebih tinggi bila dibandingkan dengan penyimpanan pada suhu yang tinggi. Penelitian ini menunjukkan penyimpanan ovarium pada suhu 4°C selama 8-10 jam dapat mempertahankan kompetensi pematangan oosit domba, lebih baik bila dibandingkan dengan penyimpanan pada suhu yang lebih tinggi.

Kata Kunci: ovarium, In vitro maturasi, oosit domba, temperatur, waktu penyimpanan

ABSTRACT

The present study was conducted to investigate the effects of duration and temperature storage of sheep ovaries on nuclear maturation *in vitro* of oocytes obtained from stored ovaries. Ovaries were collected from slaughterhouse and stored in physiological saline for 2-4 h, 5-7 h and 8-10 h at various temperatures (27-28°, 36-37° and 4°C). Cumulus oocyte complexes (COCs) were collected with slicing method from each group and matured *in vitro* for 26 h. There was no difference between the proportions of oocytes maturation to metaphase II when collected from ovaries stored at 27-28°C and 36-37°C for 2-7 h of slaughter. However, the percentages of oocytes from ovaries stored at 4°C were significantly lower (P<0.05) than those stored at higher temperature (69.23%, 70.83% and 45.65%, for 27-28, 36-37 and 4°C at 2-4 h, respectively; 59.61%, 64,58% and 36.36% for 27-28, 36-37 and 4°C at 5-7 h, respectively). The proportion of oocytes meiosis to metaphase II (MII) were significantly (P<0.05) decreased when the ovaries stored at 27-28 and 36-37°C for 8-10 h, but not in oocytes stored at 4°C (24.37%, 7.84% and 45.23%, respectively). The maturation rate of oocytes from ovary stored at 4°C was higher than those collected from ovaries stored at higher temperature (P<0.05). This finding indicated

that the storage of ovaries at 4°C for 8-10 h is effective for maintaining the development competence of sheep oocyte better than storage at higher temperature.
Keywords: ovary, in vitro maturation, sheep oocyte, temperature, time storage

INTRODUCTION

In vitro fertilization (IVF) is one of biotechnology in reproduction implementing aspiration and maturation of oocytes, fertilization by spermatozoa, and growth of embryo outside the animal body (Mamo, 2004). The ability of to growth and fertilize immature oocytes in vitro is useful for producing a large number of embryos for developmental biology, cryopreservation and genetic studies, as well as for live animal production.

The source of oocytes for embryos production in vitro can be collected from the live animal ovaries of through of ovum pick up (OPU) techniques. However, this technique requires expertise in basic techniques and the relatively high cost. Another alternative is utilize ovaries from slaughtered animals at abattoiras a source of oocytes (Gordon, 2003).

In Indonesia, sheep is a potential livestock for meat production. In 2012, the number of sheep slaughtered in abattoir and reported outside abattoir reached 291.317 heads (Statistics Indonesia, 2012). Therefore, there is an opportunity to obtain oocytes from abattoir ovaries for in vitro embryo production. However, the time interval between animal slaughtering and removing the oocytes isolation from the follicle should be noted. After slaughter, the ovary will loss the oxygen supply and energy due to interruption of blood flow which in turn puts the ovaries in ischemica conditions (Lopes *et al*., 2009). This ischemic condition triggers changes inaerobic to anaerobic metabolism thereby stimulating of lactate and proton production. The ischemic condition also triggers depolarization of the cell disruption in the balance of ions that ultimately leads to cell death (Taylor, 2006). Moreover, during the transportation of ovaries from the slaughter house to laboratory, the ovaries or oocytes exposed widely to varying temperature that may affect oocyte quality in terms of nuclear maturation and developmental competence after in vitro maturation (IVM) and fertilization (IVF).

Recent studies on abattoir ovaries have shown the developmental competence of oocytes recovered from ovaries stored at different duration temperatures of storages with various results. Matsushita *et al.* (2004) reported that storing of bovine ovaries at 10-20°C for 24 h did not affect the developmental competence of the oocytes after maturation and subsequent cleavage and blastocyst formation after IVF. In mare, ovaries can be stored for 6–8 h at temperature of 27-37°C during transportation without remarkable effects on the nuclear maturation and cytoplasmic membrane integrity of oocytes (Love *et al*., 2003). The storage of pig ovaries at 25–35°C for 6 h is effective for maintaining the developmental competence of the oocytes, however, oocytes recovered from the ovaries that stored at 4°C for 6 h are developmentally incompetent after maturation (Wongsrikeao *et al*., 2005). In goat, that storage of ovarian at 20 or 39°C significantly reduced the percentage of normal preantral follicles except preservation at 4°C (Ferreira *et al*., 2001). The storage of sheep ovarian fragments at 20-39°C for 12 and 24 h reduced the percentage of morphologically normal preantral follicles than the preservation at 4°C in all incubation times (Andrade *et al.,* 2002). The limited information is available on effects of storing ovaries interval and on the temperature sensitive of sheep oocytes after IVM. Therefore, the objective of this study was to examine the effects of ovaries storage for duration and temperatures of storage on the nuclear maturation of oocytes collected from stored ovaries after IVM in sheep.

MATERIALS AND METHODS

Collection and Maturation of Oocyte

Ovaries were collected from a local slaughterhouse. Ovaries were randomly assigned into 3 groups (at temperature of 27-28°, 36-37° and 4°C) and then transported to the laboratory in physiological saline (0.9% NaCl) supplemented with 100 IU/ml penicillin and streptomicyne. Each ovary of each group was then sliced repeatedly with a scalpel blade to release cumulus oocyte complexes (COCs) in *Phosphate Buffered Saline* (PBS) supplemented with 0.3% *Bovine Serum Albumin* (BSA) (Sigma, F-7524) at 2-4 h, 5-7 h and 8-10 h after slaughter. Only oocyte exhibiting uniform, dark pigmented ooplasm and an intact cumulus cell investment (Figure 1) were used. Collected oocytes were washed in

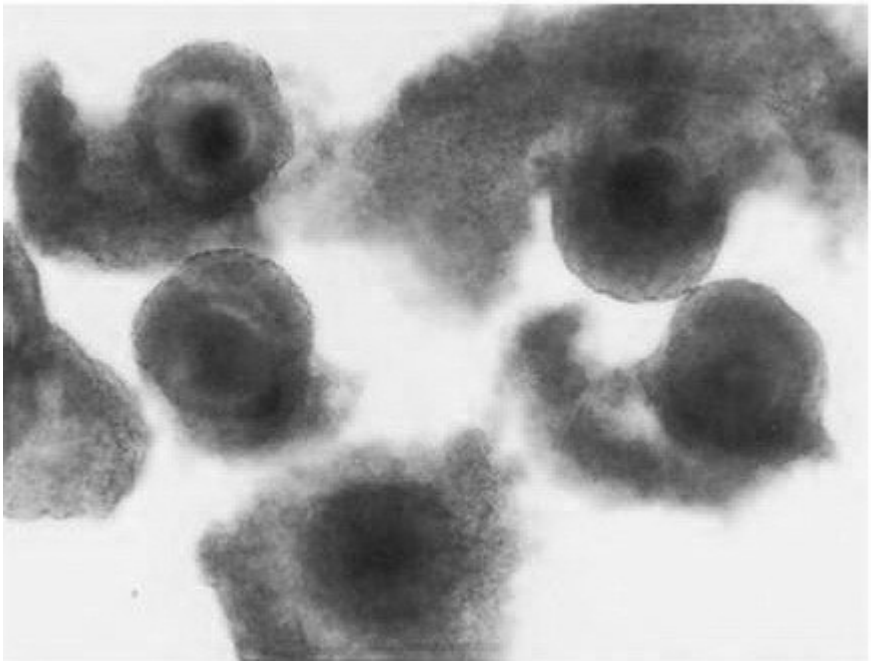

Figure 1. Cumulus Oocyte Complexes Harvested from Sheep Ovaries Obtained from a Slaughterhouse

maturation medium twice and then maturated in maturation medium was performed according to the method described by Hasbi *et al.* (2012) with minor modifications, the composition was *Tissue Culture Medium* 199 (Sigma, USA) added with 0.5% FBS, 10 IU/ml *Pregnant Mare Serum Gonadotrophin* (PMSG) (Intergonan, Intervet Deutschland GmbH), 10 IU/ml *human Chorionic Gonadotrophin* (hCG) (Chorulon, Intervet International B.V. Boxmeer-Holland), and 10 µg/ml *gentamycin* (Sigma, G-1264). Oocytes were cultured separately in 100 µl drop of maturation medium (10-15 oocytes per drop) under mineral oil (Sigma-Aldrich. Inc, M-8410) in incubator with 5% CO_2, temperature 38.5 °C for 26 h.

Assessment of Nuclear Status

At the end of IVM culture, oocytes were mechanically denuded from cumulus cells in Dulbeccos`s phosphate-buffered saline supplemented with 1 mg/mL hyaluronidase enzyme by pipetting repeatedly. The oocytes were then fixed with acetic acid ethanol (1:3, v/v) for 48-72 h. The fixed oocytes were stained with acetic-acid (1% orcein in 45% acetic acid) solution and examined under a phase contrast microscope (Olympus IX70, Japan) according to the method described by Hasbi *et al.* (2012). Nuclear maturation of oocytes was determined, based on the changes in its chromosome configuration and nuclear membrane. Status of the oocyte nucleus are grouped into stage germinal vesicle (GV), Metaphase I (MI), Anaphase-Telophase (AI/TI) and Metaphase II (MII) (Figure 2) refers to Shirazi and Sadehgi (2007).

Experimental Design and Data Analysis

This study was design with completely randomized design and the maturation rate in each group repeated for 5 times. The percentage of matured oocytes were analyzed by analysis of variance (anova) using SPSS ver 19 (SPSS Inc., Chicago, IL, USA), followed by Duncan's Multiple Range Test (DMRT) if anova revealed a significant value according to Steel *et al*. (1997).

RESULTS AND DISCUSSION

The present study were conducted to examined the ability of sheep oocytes to undergo *in vitro* meiotic maturation after recovery from ovaries stored for 2-4 h, 5-7 h and 8-10 h at temperature of 27-28°, 36-37° and 4°C. Nuclear status of in vitro matured sheep oocytes from ovaries stored before oocyte recovery in this study is presented in Table 1. There was no difference

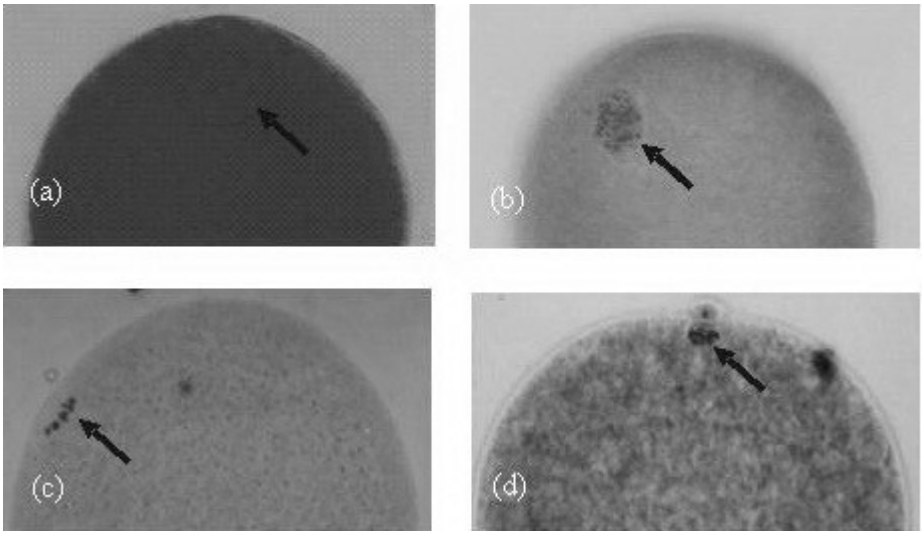

Figure 2. Different Nuclear Maturation Stages of Sheep Oocutes Fixed and Stained with 1% Orcein; (a) Germinal Vesicle; (b) Metaphase-I; (c) Anaphase; (d) Metaphase-I

between the proportions of oocytes that undergoes maturation to metaphase II when collected from the ovaries stored at 27-28 °C and 36-37 °C for 2-7 h from slaughter (69.23% and 70.83%, respectively). However, the percentages of oocytes from ovaries stored at 4 °C were significantly lower ($P<0.05$) than those stored at higher temperature (45.65%). The proportion of oocytes undergoes meiosis to MII were significantly decreased ($P<0.05$) when the ovary stored at 27-28 and 36-37 °C for 8-10 h (24.32% and 7.84%).

The data showed that storing the ovaries to a low temperature of 4 °C before recovery of oocytes may adversely affect their subsequent in vitro maturation. Therefore, it seems acceptable to transport the ovaries at more than 27-37 °C for 5-7 h for maintaining the ability to complete maturation in sheep. It is indicated that Adequate temperatures and duration for ovary storage had a subsequent influence on oocyte maturation. It has been demonstrated that ovaries were held during storage at 35 °C for 7 h did not affect equine oocyte meiotic or developmental competence (Ribeiro *et al*., 2008). Different result showed that keeping horse ovaries at 30 °C for more than 3 h or at room temperature for longer than 5 h increased the apoptosis of granulosa cells (Pedersen *et al*., 2004). Block and Hansen (2007) reported that previous reports in routine bovine IVF studies showed that the optimal ovary storage condition was 25-30 °C for 3–6 h. This was inconsistent with another study in bovine, oocytes derived from ovaries stored at a relatively low temperature (15 °C) showed higher developmental competence when compared to those from ovaries stored at control (25 °C) or high (35 °C) temperatures (Wang *et al*., 2011). Matsusthita *et al.* (2004) reported that ovary storage temperature at 10 °C for 10h had no effect on oocyte developmental competence in bovine.

The proportion of oocytes undergoes meiosis to MII decreased after 7 h in this study might cause ovaries undergo ischemic conditions during transport. When ovaries are removed from the animals, and a small change in the storage temperature or preservation solution during ovary transport had a great effect on the developmental competence of oocytes (Lopes *et al*., 2009). The storage of ovaries without blood supply might affect the oocyte quality by influencing the extracellular environment surrounding the oocytes. During transportation of ovaries to the laboratory, the occlusion of blood flow reduces the supply of oxygen and energy to the ovaries and caused them ischemic and re-oxygenationconditions. Re-oxygenationisfound to generate toxic free oxygen radicals (Moeller *et al.,* 2004) such as superoxide anion (O-), toxic hydroxylradicals (OH-), and hydrogen peroxide (H_2O_2), which react with proteins, lipids and DNA, resulting in inactivation of enzyme, membrane lipid peroxidation and DNA alteration (Catala, 2009). Storing of porcine ovaries for 6-12

Table 1. Nuclear Status of *in vitro* Matured Sheep Oocytes from the Ovaries Stored at Various Temperature for Different Storage Time

Group		Number of Oocyte	Nuclear Status (%)				
Time (h)	Temperature (°C)		GV	GVBD	MI	A/T	MII
2-4	36-37	48	1 (2.08)^{a}	2 (4.16)^{a}	10 (20.83)^{abc}	1 (2.08)^{a}	34 (70.83)^{a}
	27-28	52	1 (1.92)^{a}	1 (1.92)^{a}	12 (23.07)^{abc}	0 (0.00)^{a}	36 (69.23)^{ab}
	4	46	4 (8.69)^{a}	4 (8.69)^{a}	13 (28.26)^{bc}	0 (0.00)^{a}	21 (45.65)^{c}
5-7	36-37	48	1 (2.08)^{a}	3 (6.25)^{a}	8 (16.66)^{ab}	1 (2.08)^{a}	31 (64.58)^{abcd}
	27-28	52	4 (7.69)^{a}	2 (3.84)^{a}	9 (17.30)^{ab}	0 (0.00)^{a}	31 (59.61)^{abcd}
	4	55	6 (10.90)^{a}	8 (14.54)^{ab}	14 (25.45)^{bc}	0 (0.00)^{a}	20 (36.36)^{ce}
8-10	36-37	51	20 (39.21)^{b}	17 (33.33)^{b}	4 (7.84)^{a}	0 (0.00)^{a}	4 (7.84)^{cf}
	27-28	43	15 (40.54)^{b}	11 (29.72)^{b}	7 (18.91)^{abc}	1 (2.70)^{a}	9 (24.32)^{f}
	4	42	2 (4.76)^{a}	6 (14.28)^{ab}	14 (33.33)^{c}	0 (0.00)^{a}	19 (45.23)^{ce}

Germinal Vesicle (GV), Germinal Vesicle Breakdown (GVBD), Metafase I (MI), Anafase/Telofase (A/T), Metafase II (MII). Within each end point, column with different letters (a, b, c, d, e, f) are significantly different among the groups (P< 0.05).

h caused a decreasing in pH of follicle fluid that may induce acidosis of follicular fluid by ischemia in the ovary, leading to DNA fragmentation of oocytes in follicles (Wongsrikeao *et al.,* 2005). Decrease in pH as a consequence of lactic acidosis has been shown to injure and inactivate mitochondria. Lactic acid degradation of NADH (which is needed for ATP synthesis) may also interfere with adequate recovery of ATP levels post ischemically (Varshney *et al.,* 2007). Lactic acid could also increase the amount of free-radical mediated injury (Urso and Clarkson, 2003).

In this study, the proportion of oocytes undergoes meiosis to MII did not alter from the beginning until 8-10 h of ovary storage (P>0.05). The maturation rate of oocytes from the ovary stored at 4°C was higher (P<0.05) than those stored at higher temperature (45.23% versus 24.32% for 27-28°C and 7.84% for 36-37°C) after storage for 8-10 h. Our data indicated that sheep oocytes stored in this method had some degree of cold tolerance. Matos *et al.* (2004) reported that maintenance at 4 and 20°C preserves morphology of ovine preantral follicles for longer times than at physiological temperature. This finding was contrast to in porcine (Wongsrikeao *et al*., 2005) that oocytes recovered from the ovaries stored at 4 °C for 6 h before recovery were developmentally incompetent after maturation. However, in domestic cat, storage the whole ovaries in saline at 4 °C inhibits taphonomic changes (progressive post-mortem tissue degeneration) thus the competence of oocytes after IVM and development after IVF were maintained (Wolf and Wildt, 1996). Similar phenomenon was seen when the ovaries were stored at 4 °C in this study. Storage of ovaries at a low temperature may cause some damage on the oocyte but storage of ovaries at low temperature have delayed the accumulation of acid by product and the apoptotic processes, since the metabolism and enzymes present in warm-blooded animals work most efficiently at body temperature (Wongsrikeao *et al.,* 2005). The principle of cell preservation is ingrained in the observation that life processes are temperature dependent chemical reactions. Similar to the kinetics of every chemical reaction, the metabolism can be also reduced by lowering the temperature. Most enzymes of normothermic animals showed a 1.5 to 2-fold decrease in metabolic activity for every 10 °C decrease in

temperature, purely from kinetic predictions. A decrease in temperature from 37 to 0°C would decrease the metabolism by 12-13 folds. Consequently, the preferred method for long term cell preservation is through reduced temperatures (Rubinsky, 2003). The ability to store ovaries before IVM/IVF had profound implication for applying this technology to conservation. If intra-ovarian oocytes could remain sufficiently viable under prolonged cold conditions to allow successfully IVM/IVF, then it should be possible to rescue genes from valuable individuals post-mortem by shipping the ovarian tissue to a central facility for processing and embryo production.

CONCLUSION

Storage ovaries at more than 27 °C for 5-7 h were acceptable for maintaining the ability to complete maturation in sheep. For prolonged storage, cold storage of the whole ovary at 4 °C was better than storage at higher temperature. Further study is needed to examine the competence of stored oocytes after fertilization in vitro.

ACKNOWLEDGMENT

This study was supported in part by *Hibah Bersaing Institut Pertanian Bogor T.A. 2011* No. 15/13.24.4/SPP/PHB/2011. The authors express sincere acknowledgement to the staff of the Meat Inspection Office of Bogor City for supplying the sheep ovaries.

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